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Mode of action of marine saponins on neuromuscular tissues[1]

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ABSTRACT The steroidal saponins elaborated by members of the echinoderm family of sea animals (e.g., sea cucumbers and starfish) are characterized by the principal structural elements: *1)* a complex steroid nucleus; *2)* a series of closely related sugars attached glycosidically to the 3-position of the steroid; and *3)* a negative charge locus imparted by esterification of a sugar hydroxyl group with a sulfuric acid moiety. The anionic saponins are surfactants, and highly irreversible in their destruction of excitability at a cholinergic neuromuscular junction. Their efficacy and specificity in neuromuscular junction blockade appear to be closely linked to the polarity of the sugar residues, and to a requirement for negative charge in order to achieve maximum irreversibility in tissue interactions. Further, it has been found that the degree of their effects and their irreversibility in neuromuscular junction blockade are a sensitive function of both the total pressure under which the neuromuscular tissue operates, and of the degree of load against which the muscle works. These observations are interpreted in terms of potential sites and characteristics of the tissue chemoreceptor elements inactivated by the animal saponins. —FRIESS, S. L. Mode of action of marine saponins on neuromuscular tissues. *Federation Proc.* 31: 1146-1149, 1972.

AN INTERESTING GROUP of animal saponins is elaborated by certain poisonous species of marine echinoderms, especially among sea cucumbers (family Holothurioidea) and starfish. These toxins are found as complex mixtures of steroid glycosides in which a single hydroxyl function of a glycosylating sugar residue has generally been esterified with a sulfuric acid moiety to yield an anionic charge locus on the sugar chain.

A particularly well-studied example of this class of toxins is the principle isolated from the Caribbean sea cucumber *Actinopyga agassizi* Selenka, and crystallized to high purity in the form of a sodium salt designated as Holothurin A by Chanley and his colleagues (2). Extensive work (3) on the structure of the major aglycone residue and the sugar content of this principle leads to a provisional formulation of the structure of a representative component as in Fig. 1. The crystalline toxin, hereinafter designated as H- to emphasize its negative-charge characteristic, is seen to contain a linear array of glycosylating sugars related stereochemically to D-glucose, with the interesting inclusion of the sugar D-quinovose in which the C6 terminal carbon is fully reduced to the low-polarity $-CH_3$ function. Such terminally reduced sugars are met commonly in admixture with normal sugars in the echinoderm saponins, with notable examples to be found in toxins from the Japanese starfish *Asterias amurensis* (9, 10), which contain either D-quinovose or D-fucose (terminally reduced D-galactose). The point is of some significance, since it has been noted that the relative activity of a given saponin in blockade of a cholinergic neuromuscular preparation is in part related to the number and nature of the sugar residues attached to the steroid nucleus.

The present report is concerned with the results of studies on the interactions of these steroidal saponins with neuromuscular tissues, under normal and stressful environmental conditions.

METHODS AND RESULTS

Principal studies on the mechanisms of interaction of echinoderm saponins with a model neuromuscular tissue have employed the phrenic nerve-diaphragm preparation (PN-D),[2] from the rat, working in vitro in either isotonic or isometric mode. Basic features of the execution and use of the preparation follow the classical procedures established by Bülbring (1). Modification of the apparatus to accommodate function of tissue in a hyperbaric gaseous environment ($He/N_2/O_2/CO_2$) has been described recently (5). A further alteration of the pressure chamber interior to permit deployment of a Ringer solution of toxin in a holding funnel, and a magnetic valve device to regulate its addition to the gassed tissue bath under high pressures, was readily effected.

Early findings (4, 8) with Holothurin A and the

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[2] Abbreviations: PN D, phrenic nerve diaphragm, DeH, desulfated neutral derivative, H, Holothurin A, N twitch, indirectly elicited twitch, M-twitch, directly elicited twitch.

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13. ABSTRACT

The steroidal saponins elaborated by members of the echinoderm family of sea animals (e.g., sea cucumbers and starfish) are characterized by the principal structural elements: 1) a complex steroid nucleus; 2) a series of closely related sugars attached glycosidically to the 3-position of the steroid; and 3) a negative charge locus imparted by esterification of a sugar hydroxyl group with a sulfuric acid moiety. The anionic saponins are surfactants, and highly irreversible in their destruction of excitability at a cholinergic neuromuscular junction. Their efficacy and specificity in neuromuscular junction blockade appear to be closely linked to the polarity of the sugar residues, and to a requirement for negative charge in order to achieve maximum irreversibility in tissue interactions. Further, it has been found that the degree of their effects and their irreversibility in neuromuscular junction blockade are a sensitive function of both the total pressure under which the neuromuscular tissue operates, and of the degree of load against which the muscle works. These observations are interpreted in terms of potential sites and characteristics of the tissue chemoreceptor elements inactivated by the animal saponins.

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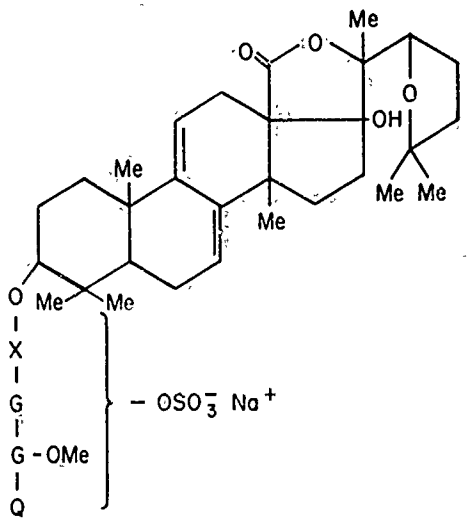
14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Steroidal saponins						
Echinoderm						
Sea cucumbers						
Starfish						
Cholinergic neuromuscular junction						
Junction blockade						
Irreversibility in tissue interactions						
Total pressure						
Degree of load						
Chemoreceptor elements						



SUGAR	SYMBOL
D-GLUCOSE	G
D-XYLOSE	X
D-QUINOVOSE	Q
3-O-METHYLGLUCOSE	G-OMe

FIG. 1. Provisional structure of the major component of Holothurin A.

PN-D preparation working in isotonic mode pointed to the high potency of the anionic saponin at an incubation level of 1.0×10^{-4} M, in an action pattern characterized by: *1*) the development of a powerful contracture which is partially alleviated in time and further relieved on washing; and 2) the fast, irreversible blockade of indirect (N=) twitch responses, with a somewhat slower reduction in amplitude of the directly elicited (M=) twitch. Further study revealed that the irreversibility of twitch blockade produced by H^- could be mitigated appreciably by preincubation of the PN-D preparation with trace concentrations of classical nonphosphorylating anticholinesterases, such as physostigmine, neostigmine, or galanthamine. At tissue bath incubation levels of these agents (e.g., 5×10^{-10} M for physostigmine) far below those at which they produce direct effects on tissue responses, a remarkable degree of concentration-related protection against the *irreversible* facets of N-twitch blockade by 1.0×10^{-4} M H^- is seen. In general, the protective effect exerted by a given protective agent (Pro^+) present in the tissue bath before addition of H^- starts at concentrations below those at which Pro^+ is antiesterasic, rises to an optimum degree of protection at a concentration (C_{opt}) characteristic of each specific protector, and falls again as the solution concentration of Pro^+ is further raised beyond its optimum or peak value. For the three protective agents tested, the values of C_{opt} appear to be related linearly to the anticholinesterasic potencies of the agents, as measured by C_{50} values for inhibition of the acetylcholinesterase acetylcholine system in vitro.

These findings led (8) to the formulation of a receptor model for sites at the rat PN-D myoneural junction susceptible to H^- attack and amenable to partial protection by charged Pro^+ protective species. This model is sketched in Fig. 2, which depicts E_1 sites as AChE or AChE-like loci, and nearby or contiguous F_1 sites as those destroyed irreversibly by attack of the anionic H^- species. In this model, the binding of Pro^+ species at E_1, and the simultaneous presence of negatively charged H^- species bound at F_1 (in a mode that normally leads to irreversible destruction at F_1), brings bound Pro^+ and H^- ions to appropriate spatial separation distance for weak ion pairing via electrostatic interaction. Such pairing is pictured as leading directly to a diminution in the strength of the H^-–F_1 interaction presumably underlying any surface alteration induced at F_1, in a sparing action just sufficient to negate the destruction of F loci by H^-, but not strong enough to cause disruption of the primary adsorption of H^- species to F loci leading to the observed *reversible* blockade of the N-twitch in the presence of Pro^+ and H^-. Finally, the model also rationalizes the finding of decrements in protective effect exerted by Pro^+ at concentrations in excess of C_{opt} by the concept that excessive numbers of Pro^+ ions bound at the E_1 surface could conceivably deform it by mutual repulsion of like charges, and disrupt the favorable contiguity between E_1 and F_1 surfaces.

The receptor model in Fig. 2 also accommodates findings (7) on reduced potency of the desulfated, neutral derivative (DeH) of the natural toxin H^-. Removal of the anionic charge center from the saponin increases the concentration causing direct contracture in the muscle, and blocking the N- or M-twitch response, by about one order of magnitude. Further, the blocking actions of DeH are largely reversible on washing, and the uncharged species at the $1–5 \times 10^{-5}$ M level is able

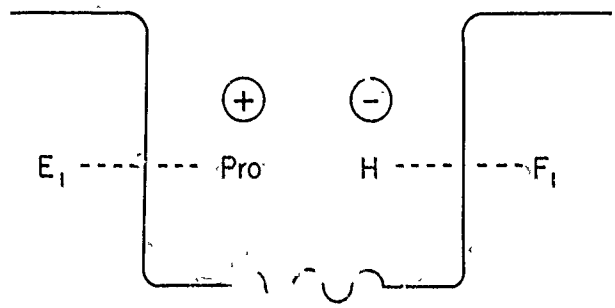


FIG. 2. Postulated model for interactions of Holothurin A and protective agent with receptor.

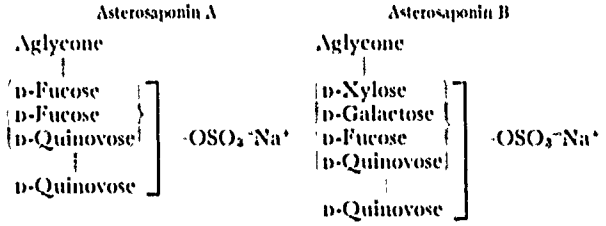


FIG. 3. Sugar contents of Asterosaponins A and B.

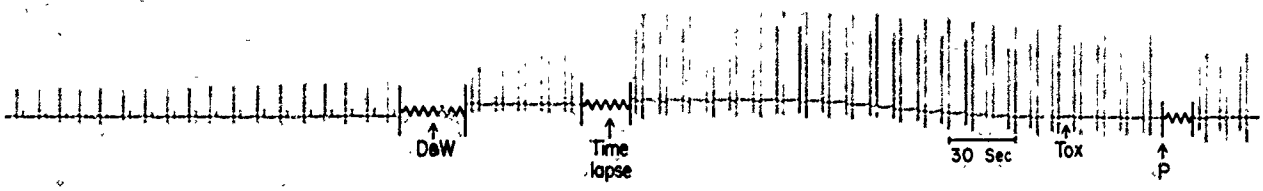


FIG. 4. Representative tracing of the action of 1.0×10^{-4} M H^- on the pressurized (330 psia) PN-D preparation.

to afford significant elements of protection against the *irreversible* destruction of twitch response normally evoked by H^- at the 1.0×10^{-4} M level in the bathing medium. These observations are in accord with the design of Fig. 2 in the sense that DeH binding at F_1 loci is blocking in character but nondestructive, and that DeH bound at F_1 by preincubation prevents in large measure the binding of H^- to F_1 in destructive mode.

The chemical nature of receptor loci in the PN-D susceptible to echinoderm saponin inactivation came under further scrutiny (6) with the aid of purified samples of two steroidal saponins, Asterosaponins A and B, isolated from the starfish *Asterias amurensis* Lutken. The two Asterosaponins, isolated and characterized in the laboratories of Hashimoto et al. (9, 10), contain the same principal aglycone moiety and a single esterifying sulfuric acid residue per molecule, but differ in the number and nature of the sugar residues attached to the steroid nucleus. As shown in the linear arrays of Fig. 3, in which the sugar positional sequences are postulated by analogy with the known sequence of Holothurin A, the Asterosaponin A contains (9) four sugars all of which are of the reduced polarity, C6-reduced type. In contrast, Asterosaponin B contains (10) a mixture of sugars, two of which are of standard sugar polarity (xylose and galactose), while the remaining three are of the C6-reduced type. The resulting difference in net polarity of the glycosidic sugar chains of the two Asterosaponins is responsible for a surprising differentiation in their apparent modes of action in PN-D blockade. Asterosaponin A (lowered polarity) is relatively more potent in blockade of indirectly elicited, isotonic contraction (N-twitches) than against responses provoked by direct muscle stimulation (M-twitches). Conversely, Asterosaponin B (higher polarity) displays an inverted activity sequence in the sense of the blockade potency inequality M-twitch $\geq$ N-twitch. It is attractive to correlate this finding with the proposition that two categories of saponin-receptor sites exist in the PN-D tissue complex which are differentially sensitive to the net surface polarity of the sugar "tail" in an attacking saponin molecule. The first category, exhibiting preferential affinity for the terminal CH_3-containing residues in the sugar content of Asterosaponin A, might well be located in the neural segment of the junction, and specifically in or near the myelin-coated tissue in nerve fibers and terminals. The second category, responsive to more highly polar adducts, could well favor Asterosaponin B by binding it preferentially at loci on the postjunctional membrane, which has been extensively documented in terms of its ability to initiate de-

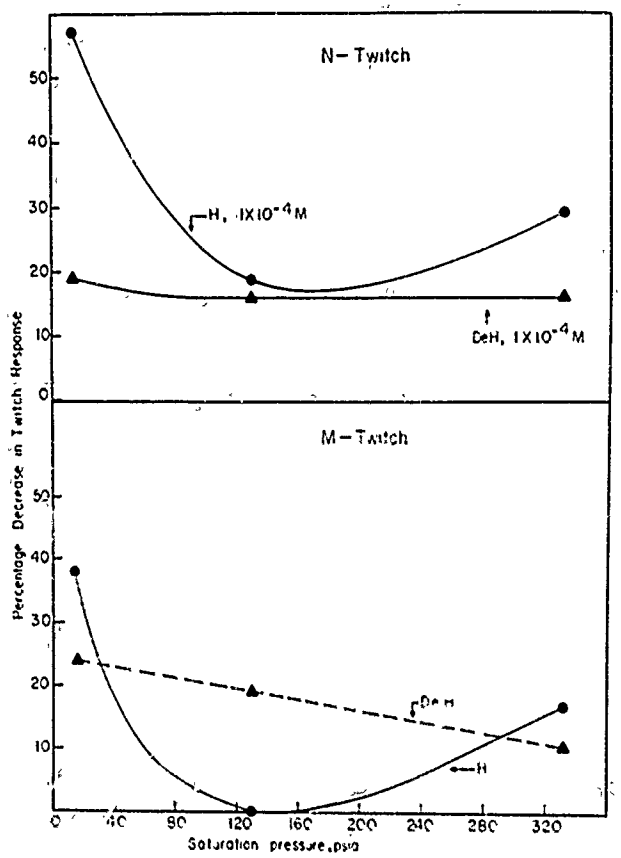


FIG. 5. Effects of change in total environmental pressure on twitch blockade indexes for H^- and DeH, 1.0×10^{-4} M, respectively, acting on the isometric PN-D preparation.

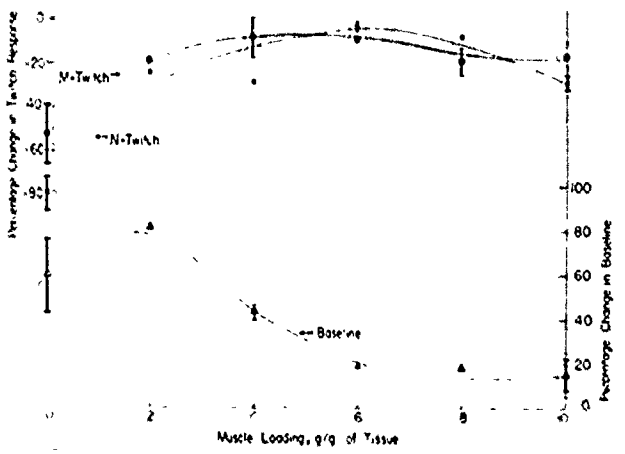


FIG. 6. Effects of muscle loading (g/g of tissue) on potency of 1.0×10^{-4} M H^- in blockade of PN-D twitch responses, and in change of base-line tension. Units on base-line tension axis are relative to control M-twitch tension = 100.

polarizing-blocking events by the binding of highly polar entities.

In another approach to the investigation of saponin-receptor interaction properties, some interesting information has been obtained recently from studies with the echinoderm saponins H⁻ and DeH and the rat PN-D preparation working in isometric mode, under normobaric (O_2/CO_2, 1 atm) and hyperbaric ($He/N_2/O_2/CO_2$) environmental conditions. As an example of response alterations observed, Fig. 4 depicts the isometric twitch responses of the PN-D preparation in an experimental sequence reading from right to left, with the first response in each twitch pair being the N-twitch, followed by the M-twitch response. After equilibration under 1 atm of (O_2 (95%), CO_2 (5%)), at point P the system was pressurized gradually to 330 psia with (He (27%), N_2 (50%), O_2 (20%), CO_2 (3%)), with no evidence of deleterious effect. At the symbol Tox, H⁻ was added to the pressurized tissue bath Ringer in sufficient quantity to bring its concentration to 1.0×10^{-4} M. The ensuing phenomena include a relatively small rise in base-line tension, and a slow but progressive decay in N- and M-twitch tensions over the course of 300–400 sec of incubation and work. At the symbol D & W, gradual decompression and Ringer wash occurred, followed by a period under flush with 1 atm of O_2/CO_2 which witnessed further decay in the twitch response tensions.

The effect of total pressure on the efficacy of H⁻ and DeH interactions with the working (isometric) PN-D preparation has been determined at the pressure levels 14.7, 130, and 330 psia, with results shown in Fig. 5. Figure 5 records the effects of change in total gaseous pressure on twitch blockade indexes for N- and M-twitch responses, at the reference time interval 270–330 sec after addition of saponin to the bath, for H⁻ and DeH at 1.0×10^{-4} M, respectively. Surprisingly, it is noted that the degree of blockade by anionic H⁻ is very pressure dependent in terms of effects on both N- and M-twitch responses: the effect of saponin falls sharply as the total pressure is increased from 14.7 to 130 psia, and then increases somewhat as the pressure is raised further to 330 psia. In contrast, the effect of pressure on the effect of DeH on PN-D twitch responses is virtually insignificant, with very slight declines in N- and M-twitch blockade potency being noted as gas pressure is increased.

The second stress factor imposed on PN-D function in isometric records such as those of Fig. 4 is that of muscle loading. It was of importance to assess separately the effects of muscle loading on the strength of saponin interactions with the rat PN-D system. A representative section of this study is shown in Fig. 6, which depicts changes in magnitude of N- and M-twitch responses and base-line tension induced by 1.0×10^{-4} M H⁻ at 1 atm pressure, relative to presaponin control levels. Values are plotted for the reference saponin incubation time of 330 sec, as a function of degree of loading (g/g of tissue) of the muscle. It is seen that the depression of both N- and M-twitch responses by 1.0×10^{-4} M H⁻ at this reference time is pronounced at zero loading of the muscle, diminishes toward zero blocking potency as the loading is increased over the range 2–6 g/g of tissue, and then increases slightly as the load reaches 10 g/g of tissue. Further, the base-line tension increase produced by H⁻ is seen to be quite sensitive to the degree of muscle loading; a relatively large increase in base-line tension evident at low loading levels (0–2 g/g of tissue) gives way to progressively smaller saponin-induced increases as the muscle load approaches 8–10 g/g of tissue.

The representative findings of Figs. 5 and 6, pointing to the effects of elevated environmental pressures and muscle loading factors on the effects of H⁻/DeH in altering PN-D response parameters, can be interpreted readily in terms of characteristics of the saponin-receptor model outlined in Fig. 2. First, the observations that initial increases in pressure and muscle loading cause a sharp drop in the effect of H⁻ as a twitch blocking agent are consonant with the picture of F_1 sites for H⁻ binding as located in a shallow surface crevasse. Initial distortion of the interior surfaces of the crevasse by pressure or loading could well diminish the degree of fit of H⁻ to the F_1 binding loci. The observation that blocking actions of DeH are less pressure sensitive than those of H⁻, and more reversible on washing, could in turn be accommodated by the possibility that primary DeH binding occurs at the top of the crevasse, in a lesser proximity to F_1 loci. Finally, the curious finding that still higher pressures (near 300 psia) restore (Fig. 5) some of the H⁻-blocking potency that is lost at 130 psia can be tentatively assessed as resulting from a continuation of the pressure-induced surface deformation leading to fuller exposure of functional F_1 loci to H⁻ attack.

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